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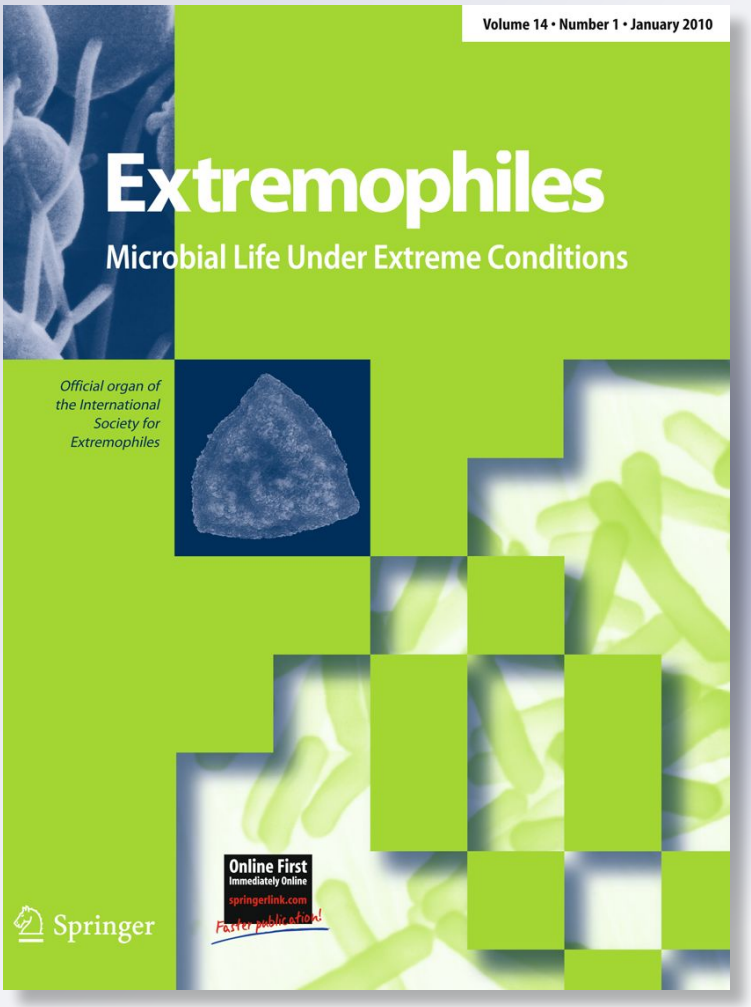

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ORIGINAL PAPER

Bacterial diversity in five Icelandic geothermal waters: temperature and sinter growth rate effects

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Abstract The microbial ecology associated with siliceous sinters was studied in five geochemically diverse Icelandic geothermal systems. Bacterial 16S rRNA clone libraries were constructed from water-saturated precipitates from each site resulting in a total of 342 bacterial clone sequences and 43 species level phylotypes. In near-neutral, saline (2.6–4.7% salinity) geothermal waters where sinter growth varied between 10 and ~300 kg year^{-1} m^{-2}, 16S rRNA gene analyses revealed very low (no OTUs could be detected) to medium (9 OTUs) microbial activity. The most dominant phylotypes found in these waters belong to marine genera of the *Proteobacteria*. In contrast, in alkaline (pH = 9–10), meteoric geothermal waters with temperature = 66–96°C and <1–20 kg year^{-1}m^{-2} sinter growth, extensive biofilms (a total of 34 OTUs) were observed within the waters and these were dominated by members of the class *Aquificae* (mostly related to *Thermocrinis*), *Deinococci* (*Thermus* species) as well as *Proteobacteria*. The observed phylogenetic diversity (i.e., number and composition of detected OTUs) is argued to be related to the physico-chemical regime prevalent in the studied geothermal waters; alkaliphilic thermophilic microbial communities with phylotypes related to heterotrophic and autotrophic microorganisms developed in alkaline high temperature waters, whereas halophilic mesophilic communities dominated coastal geothermal waters.

Keywords Bacterial diversity · Hot spring · Phylogeny · Thermophiles · Ecology · Geothermal water · Silica sinter

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Introduction

Amongst terrestrial extreme environments, geothermal hot springs and the associated silica sinters are well known analogues of habitable environments on the early Earth (e.g., Cady and Farmer 1996; Konhauser et al. 2001; Toporski et al. 2002 and references therein). They may also be analogues of hydrothermal processes on Mars as supported by the discovery of silica-rich hydrothermal deposits in Gusev Crater (e.g., Farmer and DesMarais 1999, Squyres et al. 2008 and reference therein). The microbial communities thriving in terrestrial geothermal systems have thus been the focus of extensive research.

Phylogenetic studies using 16S rRNA analysis combined with cultivation studies and in situ microbial physiological and ecological studies have shown that an abundant diversity of thermophilic microorganisms inhabit neutral to alkaline (pH 7–9), high temperature (60–95°C), silica-precipitating hot springs around the world (e.g., Japan, New Zealand, Iceland, Yellowstone National Park, USA). The bacterial communities in these geothermal waters are dominated by organisms belonging to the order *Aquificales* (e.g., Reysenbach et al. 1994; Inagaki et al. 1997; Skirnisdottir et al. 2000; Blank et al. 2002; Eder and Huber 2002; Purcell et al. 2007; Childs et al. 2008; Flores et al. 2008; Boomer et al. 2009). *Aquificales* species are

mainly obligatory chemolithotrophic, aerobic organisms that belong to one of the earliest branching orders of the domain *Bacteria*. The successful isolation of *Aquificales* species, i.e., *Thermocrinis ruber* (Octopus Spring in Yellowstone National Park, USA; Huber et al. 1998) and *Sulfurihydrogenibium kristjanssoni* (Hveragerdi, Iceland; Flores et al. 2008), suggested that primary production in these ecosystems is by chemoautotrophic hydrogen and sulphur oxidation. Other abundant organisms in these ecosystems include members of the genus *Thermus* (e.g., Brock and Freeze 1969; Kristjansson and Alfredsson 1983; Hudson et al. 1987; Chung et al. 2000; Blank et al. 2002; Hreggvidsson et al. 2006; Purcell et al. 2007; Bjornsdottir et al. 2009 and references therein). *Thermus* species are chemoorganotrophic, aerobic bacteria using organic substrates for their growth and are ubiquitous to most hot springs with slightly acidic to alkaline pH and temperatures up to 99°C (Hreggvidsson et al. 2006). These ecosystems are further characterised by species belonging to the *Bacilli*, the *Nitrospira*, the fermentative *Thermotogales*, and the sulphate-reducing *Thernodesulfobacterium* group. Similar to *Thermus*, *Bacilli* and *Nitrospira* species are chemoorganotrophic, aerobic bacteria whilst representatives of the *Thermotogales* and *Thermodesulfobacterium* are mostly anaerobic bacteria but can also use organic substrates for their growth. It is important to note that the phylogenetic analysis via sequencing also reveals many new and unknown species (e.g., new candidate divisions from Obsidian Pool, Yellowstone National Park, USA; Blank et al. 2002 and references therein), but unless new cultured representatives from these divisions can be found, it is difficult to predict the metabolic pathways they use to maintain life in these environments.

A few studies (e.g., Skirnisdottir et al. 2000; Fouke et al. 2003; Meyer-Dombard et al. 2005; Purcell et al. 2007; Childs et al. 2008; Petursdottir et al. 2009) have tried to link the diversity of microbial communities with physico-chemical conditions of the studied geothermal waters. Overall, these studies revealed that the complexity of the metabolic framework and the microbial community structure correlate well with specific geochemical parameters, including temperature, salinity, pH, and availability of energy sources. Other studies (e.g., Hreggvidsson et al. 2006; Takacs-Vesbach et al. 2008 and references therein) indicated that historical factors (e.g., climate events, sea-level changes, volcanic eruptions) and geographical barriers need also be considered, as in some cases they can explain variations in microbial community structure significantly better than contemporary environmental conditions. These observations demonstrate that the geobiology of near-boiling silica-precipitating hot springs is very complex and that there is a need for further analogue studies to obtain a more in depth understanding of the biodiversity pattern found in such ecosystems. Furthermore, these environments are key study sites in terms of biosilicification and fossilisation processes on modern Earth. Therefore, knowing what are the dominant microbial communities in these settings is essential for the interpretation of biosignatures from the Early Earth as well as elsewhere (e.g., formation of hydrothermal silica deposits on Mars).

Here, we present a detailed analysis of the composition and distribution of the bacterial communities associated with silica sinters from five different Icelandic geothermal systems whose morphologies, textures and structures were described in detail in Tobler et al. (2008). In the present study, standard molecular techniques that targeted both bacterial and archaeal 16S rRNA were employed and five bacterial clone libraries were derived. The diversity of bacterial communities was determined for each site and subsequently analysed in terms of how the major geochemical factors (e.g., temperature, sinter growth rate) affected the microbial community structure. Our data were then compared and contrasted with other molecular studies on bacterial diversities in Icelandic and other silica-precipitating hot springs around the world.

Materials and methods

Geochemistry and sinter characteristics of sampling sites

The geographical distribution and the main geochemical, morphological and hydrodynamic characteristics of the studied sites can be found in Tobler et al. (2008). In both the Tobler et al. (2008) and the current study samples were collected from spring and drain waters at 5 distinct localities, i.e., Geysir geothermal area (GY1 and GY2), Hveragerdi wastewater drain (HV), Krafla Power Station wastewater drain (KF), Svartsengi Power Station wastewater pool (SV) and Reykjanes Power Station wastewater drain (RK). The geochemical conditions of the studied geothermal waters varied considerably between sites (Fig. 1, table) which resulted in extreme variations in sinter growth rates (between 0.2 and >300 kg year^{-1} m^{-2}) and sinter morphologies (Fig. 1, in situ precipitated on glass slides).

At Reykjanes (RK), where the geothermal waters exhibited near-neutral pH, high salinity, high-temperature (high-T) and the highest total silica contents of all studied sites, the sinter growth rates were also the highest (Fig. 1, table). These physico-chemical conditions led to the formation of highly hydrated and porous, but homogeneously structured sinters made of aggregates of silica nanoparticles that developed predominantly subaqueously (Fig. 1,

Locality:	KF	GY1	GY2	HV	SV	RK
T(°C):	80	83 ± 13	79 ± 3	70 ± 4	42	75
pH:	10	9	8.7	9.1	7.7	7.5
SiO_2 (ppm):	603	363	372	304	250	695
salinity (%):	<0.1	<0.1	<0.1	<0.1	2.6	4.7
sinter growth: ($kg\ y^{-1}m^{-2}$)	19.5 ± 2.4	0.3 ± 0.1	0.7 ± 0.3	2.1 ± 0.7	9.7 ± 3.5	305 ± 20

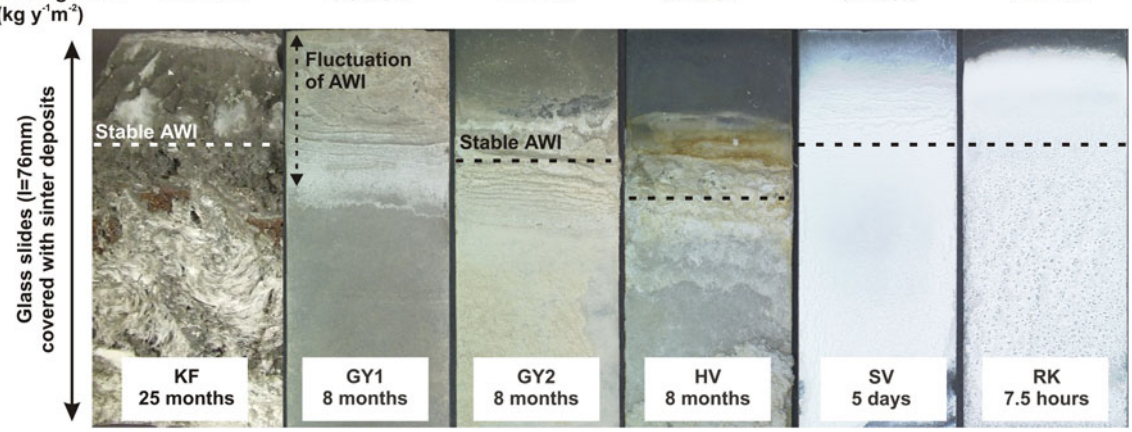

Fig. 1 Summary of physico-chemical conditions, sinter growth rate and sinter morphology at the six study sites. The air–water interface (AWI) was stable at all sites except GY1. Full details about sampling sites/intervals and further characteristics are given in Tobler et al. (2008)

RK glass side at right). Interestingly, high-resolution scanning electron microscopy (SEM) of the in situ precipitated aggregates revealed a complete lack of microbial cells amongst the porous precipitates. Note that due to the high precipitation rates, the sinter growth study had to be stopped after 5 days at this site (Fig. 10 in Tobler et al. 2008). This short sampling period might explain the lack of microbial cells in in situ grown sinters at Reykjanes.

At Svartsengi (SV), the geothermal waters exhibited significantly lower T and salinity compared with RK (Fig. 1, table), and they were undersaturated with respect to amorphous silica leading to significantly lower sinter growth rates (Fig. 1, table). When compared with RK, the precipitated silica particles were far smaller at SV, and the aggregates were more fragile and formed a gel-like surface coating on the slides (Fig. 1, SV glass slide). Similarly to RK, SEM analyses revealed again a total absence of microscopically distinguishable microbial cells associated with the precipitates. In addition, this could be due to the short sampling period at this site (between 5 days and 1 month, Tobler et al. 2008).

Conversely, at Geysir (GY1 and GY2) and Hveragerdi (HV) the geothermal waters were dominated by alkaline pH, low salinity (mostly meteoric water compositions), and high-T (Fig. 1, table). At all these sites, the geothermal waters were undersaturated with respect to amorphous silica and subaqueous precipitation was inhibited. As a result, sinter growth was mostly restricted to the air–water interface (AWI), which led to the formation of dense and heterogeneously structured sinters (Fig. 1, middle 3 slides). Despite the relatively high temperatures (66–96°C), at all three sites extensive biofilms developed in the submerged zones and at the AWI after only 5 days which, with time, became fully silicified.

Finally, the geothermal waters at Krafla (KF) were the most alkaline (pH = 10), they were of low salinity and high-T (Fig. 1, table), again resulting in highly silica undersaturated waters. Nevertheless, compact subaqueous sinters formed which consisted predominantly of silicified microorganisms. The observations at GY, HV and KF suggested that the presence of thick microbial biofilms enhanced sinter growth within the geothermal waters by acting as a template for the adhesion of suspended silica nanoparticles (Tobler et al. 2008).

Sampling and 16S rRNA gene sequence analysis

At all sites, water-saturated precipitates from the bottom of the outflow channels/pools (adjacent to the tray containing the glass slides; for more details see Tobler et al. 2008) were sampled aseptically in sterile vials. This was done under the assumption that the precipitates harbour the microbiota representative for each studied water and to have a better control and a clear link between the microbial diversity and the variations in geochemical/hydrodynamic regime and sinter growth rate between the sites. DNA extractions were carried out using the FastDNA®SPIN Kit for Soil (Q-BIOgene; combined with bead-beating using 0.1-mm silica beads) according to the manufacturer's instructions. DNA products were then PCR amplified for

bacterial and archaeal 16S rRNA genes. PCR reaction mixtures (50 µl) contained 1 µl of extracted DNA, 1× PCR enhancer (BIOLINE), 1× NH_4 buffer (BIOLINE), 1.5 mM $MgCl_2$ (BIOLINE), 0.1 mM dNTP each, 1 U *Taq* RNA polymerase (BIOLINE) and 0.5 µM of a specific bacterial or archaeal primer set. For bacterial PCR, primers 9f (5′-GAG TTT GAT CMT GGC TCA G-3′, M = A/C) and 1492b (5′-ACG GYT ACC TTG TTA CGA CTT-3′, Y = T/C) were used whereas for archaeal PCR, primers Ar109f (5′-ACK GCT CAG TAA CAC GT-3′, K = G/T) and Ar912r (5′-CTC CCC CGC CAA TTC CTT TA-3′) were employed. PCR amplifications of 16S rRNA genes were performed with a Corbett Research Palm-Cycler using a initial denaturation step at 94°C for 5 min and then 33 cycles at 94°C for 45 s, 48°C for 1 min and 72°C for 2 min followed by a final elongation at 72°C for 5 min. The bacterial PCR products were purified prior to ligation using PureLink™ PCR Purification Kit (Invitrogen), whilst archaeal PCR products were not further processed.

Bacterial PCR products were cloned into the pCR2.1-TOPO plasmid and used to transform chemically competent OneShot MACH1™ T1R *Escherichia coli* cells as specified by the manufacturer (TOPO TA cloning kit, Invitrogen). Positively transformed clones (white colonies) were then picked using a sterile toothpick and the plasmid inserts screened using colony PCR with M13f/r primers (5′-GTAAAACGACGGCCAG-3′ and 5′-CAGGAAACAG CTATGAC-3′, respectively). A touchdown cycle was chosen with 20 touchdown steps from 62–55°C and 15 further cycles at 52°C annealing temperature.

Groups of clones were subdivided on the basis of restriction fragment-length polymorphism (RFLP) analysis following *Msp*I and *Hin*6I digests. Digests were run on a 3% agarose gel to identify different 16S rRNA gene sequences from the amplified clonal inserts. Triplicates of unique restriction patterns, where possible, were purified using PureLink™ PCR Purification Kit (Invitrogen) and then sent to the Center for Genomics, Proteomics, and Bioinformatics Research Initiative (CGPBRI), University of Hawaii at Manoa (USA) for sequencing (ABI 3730XL capillary-based DNA sequencers).

Partial sequences were manually checked for ambiguities and assembled using Sequencher 4.7 (Gene Code Corporation). Contiguous sequences were then submitted to the online analyses Bellerophon 3 (Huber et al. 2004) and CHIMERA_CHECK v.2.7 (Cole et al. 2003). Putative chimeras were excluded from subsequent analyses. Non-chimeric sequences were uploaded to the Ribosomal Database Project-II (RDP-II; Cole et al. 2007 and references therein) in which all sequences were aligned to an existing alignment containing >100,000 nearly full-length bacterial sequences. Closest relatives were found using BLAST 2.2.21 search tool (Altschul et al. 1990) and RDP-II SEQMATCH. Phylogenetic trees were constructed using maximum likelihood in the PhyML-software package (Guindon and Gascuel 2003). Phylogenetic groupings were then illustrated using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Nearly full-length 16S rRNA gene sequences were grouped into operational taxonomic units (OTUs) using a ≥98% sequence similarity cut-off value in the DOTUR software (Schloss and Handelsman 2005). The output files created by DOTUR were further used to construct rarefaction curves. Biodiversity within each bacterial clone library was also estimated via the Shannon–Weaver index (Shannon and Weaver 1949) and the Chao1 index (Chao 1984). The Shannon–Weaver index, *H*, weights individual classes by their relative abundances and hence incorporates both richness (*S*) and evenness (*E*) of the studied clone libraries whilst the Chao1 index is a nonparametric estimator for expected richness only.

The 16S rRNA gene sequences determined in this study were submitted to the GenBank database (accession numbers: GU233809–GU233815, GU233821 and GU233825–GU233850).

Results

Community structure analysis

Archaeal and bacterial DNA was successfully extracted and amplified from both Geysir sites (GY1 and GY2) and from Hveragerdi (HV) whilst only bacterial DNA could be amplified at Krafla (KF). Although the microscopic evaluation did not reveal any microbial cells within the Svartsengi (SV) sinter deposits (Tobler et al. 2008), both archaeal and bacterial DNA was successfully amplified from the sample at Svartsengi. In contrast, at Reykjanes (RK) despite multiple attempts neither archaeal nor bacterial DNA could be extracted from the collected precipitates. This supported the SEM results (Tobler et al. 2008) confirming that at the bottom of the RK outflow channel microbial abundance was low (if any microorganisms were present at all), most likely due to the extremely fast precipitation combined with the high salinity and high temperature of the waters. Note that archaeal PCR products were not further processed, as the focus of this study was on the bacterial diversity only.

Bacterial PCR products from all extractions were pooled (2–3 DNA extractions were carried out per sample) and bacterial clone libraries were constructed for GY1 (61 clones), GY2 (87 clones), HV (67 clones), KF (46 clones) and SV (81 clones). A detailed inventory of the bacterial OTUs detected in each 16S rRNA clone library is given in Table 1.

Table 1 Summary of bacterial OTUs detected in 16S rRNA gene clone libraries at GY1, GY2, HV, KF and SV. For each OTU, the closest GenBank match (based on RDP-II SEQMATCH and BLAST search tool) with accession number and percentage sequence similarity, class-level affiliation, and the number of related clones are given

OUT	Inferred bacterial class of OTU	Closest GenBank match (% identity, Accession No.)	No. of clones
GY1 (3 OTUs/61 clones)			
GY1-1	*Aquificae*	*Thermocrinis albus* DSM 14484 (99–100%, CP001931)	58
GY1-2	*Bacilli*	*Geobacillus stearothermophilus* (99%, AY491497)	2
GY1-3	*Gammaproteobacteria*	Uncultured bacterium BANW657[a] (99%, DQ264605)	1
GY2 (5 OTUs/87 clones)			
GY2-1	*Deinococci*	*Thermus antranikianus* str. HN3-7T (99–100%, Y18411)	50
GY2-2	*Aquificae*	*Thermocrinis albus* DSM 14484 (99-100%, CP001931)	26
GY2-3	*Bacilli*	*Bacillus* sp. Tibet-S2a2 (99–100%, DQ108401)	4
GY2-4	*Bacilli*	*Bacillus* sp. KSM-KP43 (99%, AB055093)	3
GY2-5	Unaffiliated bacterium	Uncultured *Thermotogales* clone YNP_SBC_BP4_B47[b] (97%, AF352534)	4
HV (17 OUTs/67 clones)			
HV-1	*Deinococci*	*Thermus scotoductus* SA-01 (99%, CP001962)	33
HV-2	*Deinococci*	*Thermus islandicus* str. PRI-2268 (98–99%, EU753248)	3
HV-3–HV-4	*Deinococci*	*Thermus* sp. SRI-96 (95-97%, AF255590)	3
HV-5	*Deinococci*	*Thermus* sp. Y55-10 (98%, AF407746)	1
HV-6	*Aquificae*	*Sulfurihydrogenibium kristjanssoni* (99%, AM778960)	7
HV-7	*Aquificae*	*Thermocrinis albus* DSM 14484 (99%, CP001931)	1
HV-8	*Nitrospira*	Uncultured bacterium S_d11[c] (92–99%, HM595393)	2
HV-9	*Gammaproteobacteria*	*Stenotrophomonas* sp. CH37-11 (98%, EU057885)	2
HV-10–HV-11	*Betaproteobacteria*	*Variovorax* sp. WPCB174 (90–99%, FJ006917)	2
HV-12	*Bacilli*	*Geobacillus stearothermophilus* (100%, AY491497)	1
HV-13–HV-15	Unaffiliated bacterium	*Candidate division OP1* clone OPB14[b] (89–97%, AF027045)	10
HV-16	Unaffiliated bacterium	Uncultured bacterium S2R–162[b] (99%, FN545885)	1
HV-17	Unaffiliated bacterium	No significant result (<90%)	1
KF (9 OTUs/46 clones)			
KF-1	*Gammaproteobacteria*	*Pseudomonas mandelii* (*T*) (99–100%, AF058286)	18
KF-2	*Gammaproteobacteria*	*Gamma proteobacterium* SOC A20(46) (98%, DQ628970)	2
KF-3	*Gammaproteobacteria*	*Acinetobacter johnsonii* strain CAI-6 (99%, DQ257426)	1
KF-4	*Actinobacteria*	*Arthrobacter* sp. EM5 (99–100%, FJ517625)	9
KF-5	*Flavobacteria*	*Flavobacterium* sp. WB3.4-82 (99–100%, DQ515962)	8
KF-6	*Aquificae*	*Thermocrinis albus* DSM 14484 (99–100%, CP001931)	4
KF-7	*Alphaproteobacteria*	*Caulobacter* sp. DSM 6811 (99%, AJ227789)	2
KF-8	*Alphaproteobacteria*	*Glacier bacterium* FXI13 (99%, AY315163)	1
KF-9	*Betaproteobacteria*	*Ralstonia* sp. HI3 (99%, EF554889)	1
SV (9 OTUs/81 clones)			
SV-1	*Gammaproteobacteria*	*Marinobacter* sp. NT N31 (99–100%, AB166980)	63
SV-2	*Alphaproteobacteria*	*Sphingopyxis* sp. ZS1-22 (99–100%, FJ889655)	5
SV-3	*Alphaproteobacteria*	*Oceanicaulis alexandrii* C116-18 (100%, AJ309862)	2
SV-4	*Alphaproteobacteria*	Uncultured bacterium clone GBII-42[b] (93%, GQ441313)	1
SV-5	*Flavobacteria*	*Flavobacterium* sp. GSW-R14 (99%, GQ370387)	4
SV-6	*Cyanobacteria*	Uncultured bacterium clone Prehnite21[d] (92%, FJ230820)	3
SV-7	*Cyanobacteria*	Uncultured bacterium clone Prehnite43[e] (91%, FJ230822)	1
SV-8	*Sphingobacteria*	Uncultured bacterium ZBAF1-D5[f] (98%, HQ681988)	1

Table 1 continued

OUT	Inferred bacterial class of OTU	Closest GenBank match (% identity, Accession No.)	No. of clones
SV-9	*Sphingobacteria*	*Thermonema rossianum* str. SC-1 (99%, Y08957)	1

[a] Next closest cultured species: *Acinetobacter johnsonii* (99%, AF188300), *Gammaproteobacteria*

[b] No significant results for next closest cultured species (<90% identity)

[c] Next closest cultured species: *Nitrospira* sp. SRI-9 (92–99%, AF255603), *Nitrospira*

[d] Next closest cultured species: *Microcoleus chthonoplastes* SAG 2210 (91%, EF654045), *Cyanobacteria*

[e] Next closest cultured species: *Microcoleus chthonoplastes* EBD (91%, EF654031), *Cyanobacteria*

[f] Next closest cultured species: *Spingobacterium sp. P-7* (92%, AM411964), *Sphingobacteria*

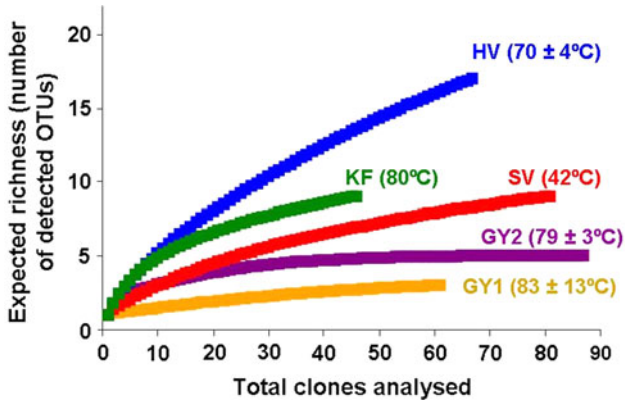

Fig. 2 Rarefaction curves for the five bacterial 16S rRNA clone libraries. The cumulative number of species (i.e., detected OTUs) was plotted against the cumulative number of individuals (i.e., clones) analysed

Rarefaction analysis was used to compare species richness and diversity between the five constructed clone libraries. The rarefaction curves indicated that most bacterial clone libraries were sampled nearly to saturation, except for SV and HV (Fig. 2). Thus, at SV and HV additional sampling of clones could potentially have resulted in a larger number of species detected (i.e., higher richness). This was also illustrated by calculated Chao1 values (i.e., estimated species richness) which were almost identical to the detected species richness (*S*) for the GY1, GY2 and KF clone libraries, but notably higher for the HV and SV clone libraries (Table 2). Despite incomplete sampling, the rarefaction curves indicated that the bacterial richness increased with decreasing *T* of the study site (except SV). This is in good agreement with previous studies (e.g., Skirnisdottir et al. 2000; Blank et al. 2002; Fouke et al. 2003; Meyer-Dombard et al. 2005) which showed a higher number of phylotypes at lower temperatures. At SV, the number of detected OTUs was significantly lower than one would have expected from the water temperature which indicated that other parameters (e.g., high sinter growth rate) limited the species richness at this site (see "Discussion").

The evaluation of the biodiversity within each bacterial clone library revealed Shannon–Weaver indices (*H*) ranging between 0.23 for GY1 and 1.92 for HV (Table 2). *H* values determined for the GY2, KF and SV clone libraries lied in between values found at GY1 and HV (1.08, 1.73 and 0.95, respectively). For comparison, *H* values from similar Icelandic and other hot springs environments were also included in Table 2.

The class-level diversity and distribution of the bacterial OTUs identified in the bacterial 16S rRNA clone libraries from GY1, GY2, HV, KF and SV is illustrated in Fig. 3. Although the columns show the percentages of each class in the total library, they do not necessarily provide a quantitative representation of the bacterial diversity within the studied geothermal systems. Nevertheless, the columns indicated that the class distribution and diversity varied substantially between sites. None of the detected classes was ubiquitous to all sampling sites but certain classes were found at more than one study site (e.g., *Aquificae* and *Gammaproteobacteria*; were both found at 4 out of 5 sites). Note that two out of five sites contained unaffiliated bacterial clones.

Phylogenetic analysis of bacterial clones

As shown by the phylogenetic inferences from the bacterial OTUs (Table 1; Fig. 3), the majority of the analysed sequences were affiliated to *Aquificae*, *Deinococci*, *Bacilli* and *Gammaproteobacteria*.

Aquificae was well represented at all sites with water temperatures above 50°C, i.e., GY1, GY2, HV and KF (Table 1). Phylotypes from each of these sites branched into the genus *Thermocrinis* and were closely related to the Iceland clone sequences SRI-48 (from hot spring microbial mats; Skirnisdottir et al. 2000) and pIce1 (from a blue filament community of a thermal spring; Takacs et al. 2001) which all represent apparent subspecies of *Thermocrinis albus*, the filament forming hyperthermophiles isolated from white streamers in the Hveragerthi area, Iceland (Eder and Huber 2002; Fig. 4). Clones belonging to

Table 2 Shannon–Weaver (*H*) and Chao1 indices for each bacterial 16S rRNA gene clone library along with the total number of analysed clones (*N*), the total number of identified species (*S*) and the evenness (*E*). For comparison, *H* values were also calculated for bacterial clone libraries from other alkaline, silica-precipitating hot springs environments

Sample	*N*	*S*	*E*	Chao1	*H*
GY1	61	3	0.21	3.5	0.23
GY2	87	5	0.67	5	1.08
HV	67	17	0.68	34	1.92
KF	46	9	0.79	11	1.73
SV	81	9	0.43	17	0.95
Olkelduhals hot spring, Iceland (Hjorleifsdottir et al. 2001)					0.55
Octopus Spring, YNP, USA (Blank et al. 2002)					0.71–1.09
High/low-sulphur mat, Iceland (Skirnisdottir et al. 2000)					1.84–1.96

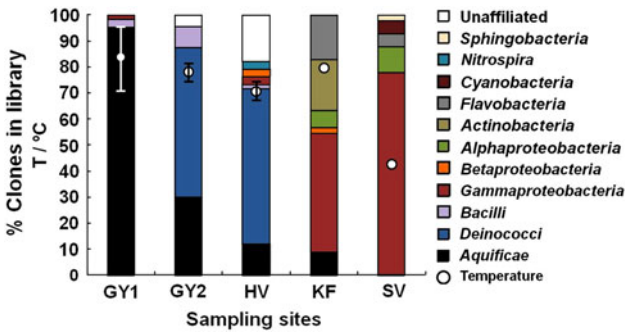

Fig. 3 Class-level distribution and diversity of 16S rRNA gene sequences within bacterial clone libraries for both Geysir sites and Hveragerdi (sites with pH ~9 and low salinity), Krafla (pH ~10, low salinity) and Svartsengi (pH ~7.7, high salinity). For reference, the temperature conditions are also given for each site

the genus *Sulfurihydrogenibium* were only identified at HV and were closely related to the Iceland clone sequence SRI-240 (from an Icelandic high sulphide mat, Skirnisdottir et al. 2000; Fig. 4). These clones also showed similarities to NAK9 from a high sulphide mat in Japan (Yamamoto et al. 1998), and to YNP-SSp_B90 from Sylvan Spring in YNP (Meyer-Dombard et al. 2005), although these two latter are not represented in Fig. 4. All these strains affiliated to *Sulfurihydrogenibium kristjanssoni (T)*, a hydrogen and sulphur-oxidising thermophile isolated from the outflow channel ($T = 68°C$, pH = 6.0) of a hot spring near Hveragerdi in Iceland (Flores et al. 2008; Fig. 4).

Deinococci representatives were only found at GY2 and HV (Fig. 3; Table 1) and all clones clustered into the genus *Thermus*. GY2 clones had the closest database match to *Thermus antranikianii* HN3-7T (Fig. 4), an Icelandic isolate that grows in alkaline waters (up to pH 10) and at temperatures around 80°C (Chung et al. 2000). In contrast, *Thermus* clones found at HV were far more diverse and affiliated to several lineages within the *Thermus* genus, including *Thermus scotoductus* (isolated from hot tap water in Iceland; Kristjansson et al. 1994), *Thermus islandicus* (isolated from hot springs in the Torfajokull geothermal area, Iceland; Bjornsdottir et al. 2009) and *Thermus igniterrae* (isolated from high-T alkaline hot springs in Iceland; Chung et al. 2000). A few HV phylotypes (e.g., OTUs HV3 and HV4) may represent novel lineages within the genus *Thermus* as indicated by the absence of any close relatives (Fig. 4).

Similarly to *Aquificae*, clones related to *Bacilli* were only found at the high-T sites, i.e., GY1, GY2 and HV (except KF; Table 1). The GY2 clones branched into the genus *Bacillus* and *Marinibacillus* and were most closely related to KSM-KP43 (an alkaliphilic *Bacillus* strain from Japan) and to Tibet-S2a2 (an alkaliphilic psychrotolerant strain from the Qinghai–Tibet Plateau), respectively (Fig. 4). In contrast, *Bacilli* clones identified at GY1 and HV belonged to the genus *Geobacillus* and were most closely related to *Geobacillus stearothermophilus* (Fig. 4), a thermophile widely distributed in soil, hot springs, and ocean sediments (e.g., Nazina et al. 2001; Derekova et al. 2008).

Members of *Alpha-*, *Beta-* and *Gammaproteobacteria* were the predominant classes at KF (54% of the clones) and SV (88% of the clones). This was particularly true for the *Alpha-* and *Gammaproteobacteria* (Fig. 3; Table 1). Note that only few *Proteobacteria* related sequences were detected in the GY1 (1 clone) and HV (4 clones) clone libraries whilst none were detected in the GY2 clone library (Fig. 3; Table 1).

Alphaproteobacteria related phylotypes identified at KF branched within *Brevundimonas* and were most closely related to isolates from both freshwater (e.g., glacier bacterium FXI13) and saline environments (*Caulobacter*, DSM6811, Fig. 4). In contrast, SV clones were more diverse and belonged to the genus *Sphingpyxis* and *Oceanicaulis* with closest relatives commonly found in seawater and salt marshes. Overall, very few *Betaproteobacteria* related sequences were detected in any of the studied sites (i.e., two clones at HV and one clone at KF,

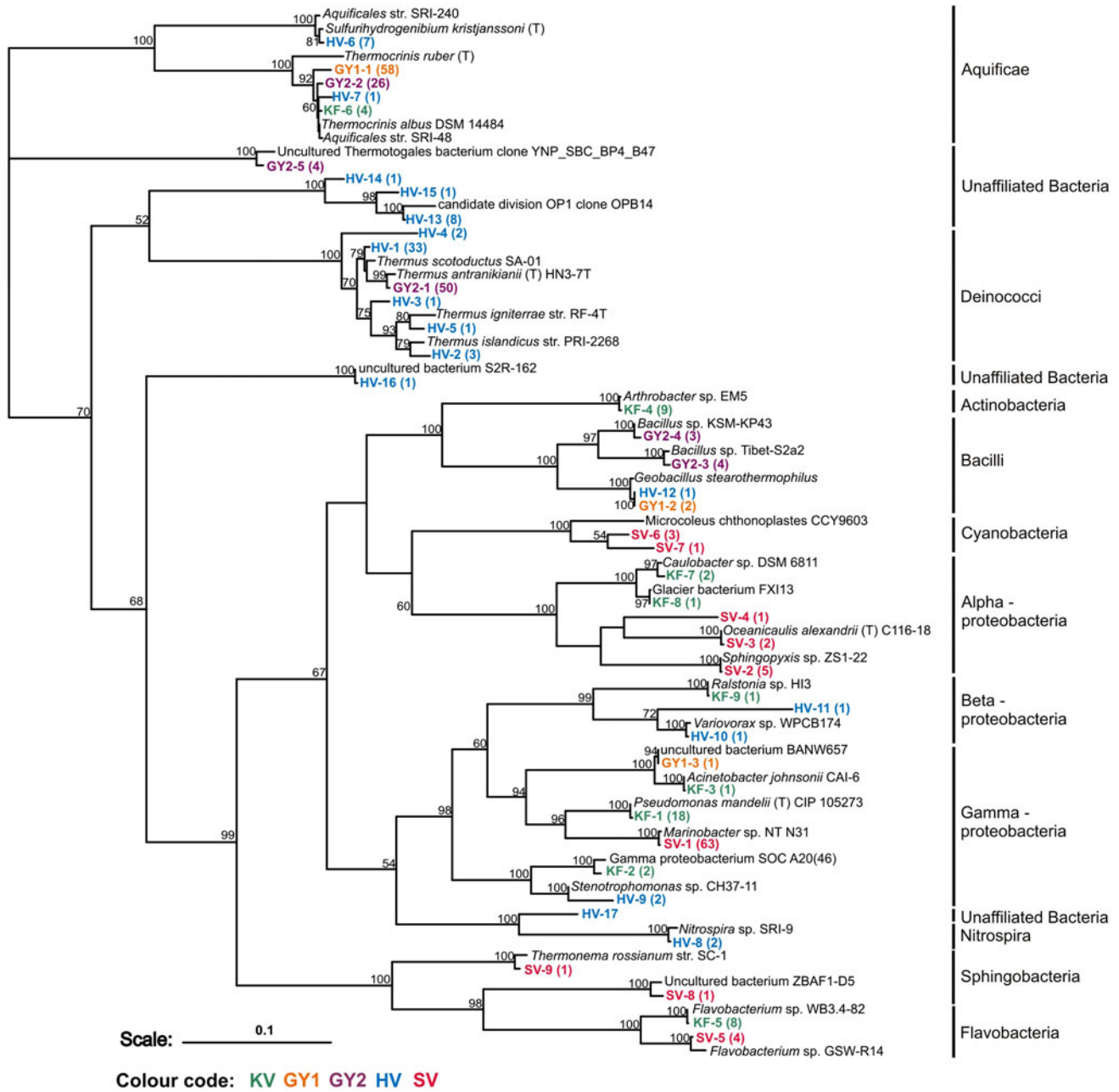

Fig. 4 Maximum likelihood tree with bacterial 16S rRNA gene sequences detected in this study in the context of currently recognised bacterial divisions. *Aquificae* related phylotypes were used as an outgroup. The *scale bar* is in nucleotide substitution per sequence position. OTUs detected in this study in *bold* with the amount of clones associated in *brackets* (Table 1). Only bootstrap values higher than 50% are shown

Fig. 4). *Gammaproteobacteria* clones were numerically the most abundant phylotypes found in the KF and SV clone libraries (Table 1). Clones that branched in the genus *Marinobacter* (i.e., genus of *Proteobacteria* found in sea water) were solely observed at SV (Fig. 4) which was not unexpected due to the high salinity of the SV geothermal waters and the proximity to the coast. In contrast, KF clones were most dominant in the genus *Pseudomonas* but also related to isolates within the genus *Lysobacter* and *Acinetobacter*. Note that *Pseudomonas*, *Acinetobacter* and *Lysobacter* have a widespread occurrence in nature (e.g., water, soil, plants; Madigan and Martinko 2005).

Finally, a subset of the phylotypes related to *Flavobacteria*, *Cyanobacteria*, *Sphingobacteria*, *Nitrospira*, and *Actinobacteria* were primarily found at SV and/or KF (except *Nitrospira* at HV, Table 1) and details on their

phylogenetic inference are given in Fig. 4. Note that unaffiliated bacterial OTUs (similarity less than 90% to known isolates) found in the GY2 and HV bacterial clone libraries (Table 1) were also included in the phylogenetic tree (Fig. 4) Further note that OTUs HV13 to HV15 were similar to the OP1 clone OPB14, a thermophilic isolate from Obsidian Pool in Yellowstone National Park (Hugenholtz et al. 1998). This group may represent a new phylogenetic class; however, for a more definite placement of this group, new cultivable representatives are needed.

Discussion

The molecular phylogenetic approach applied in this study has several potential biases (e.g., PCR-bias such as preferential amplification, different susceptibility to cell lysis, analysis of non-indigenous strains; e.g., Sambrook et al. 1989; Ward et al. 1997; Hurst et al. 2002; Fouke et al. 2003) which need to be kept in mind during data interpretation. In addition, if the number of clones or sequences is not high enough, the microbial diversity of the studied environment are often not fully represented within the constructed clone library (e.g., HV and SV in Fig. 2). However, although this method does not provide a fully quantitative picture of the microbial diversity, it gives a reliable first estimate of the microbial community structure (e.g., Reysenbach et al. 1994; Hugenholtz et al. 1998; Skirnisdottir et al. 2000; Fouke et al. 2003).

Effects of geochemical parameters on bacterial diversity

An advantage of the current study is that the geochemical and hydrodynamic regime at each study site has been quantitatively assessed in detail before (Fig. 1; Tobler et al. 2008). Thus, the results presented here (i.e., information on bacterial community structure and diversity) could be placed in a quantitative physical and chemical environmental context.

The microbial diversity in precipitates from all six study sites was analysed, but despite multiple repeat trials neither bacterial nor archaeal DNA could be extracted from the RK samples. This suggested that the physico-chemical conditions at this site, i.e., 4.7% salinity, near-neutral pH, dynamic flow, high silica concentrations (695 ppm SiO_2), and consequently very fast sinter growth rates ($\sim$300 kg year^{-1} m^{-2}, thickness of sinter accumulation $\sim$6.4 mm per day, Tobler et al. 2008) outpaced microbial growth at this site, and hence no thermophilic microbial communities were established.

The bacterial community found in the 80°C geothermal waters at KF was somehow surprising, since most of the detected phylotypes (except KF6) were closely related to well-known mesophilic species commonly found in freshwater and soils (e.g., *Pseudomonas*, *Caulobacter*, and *Acinetobacter*). Only one KF phylotype (KF6) affiliated to a thermophilic organism (*Thermocrinis albus* DSM 14484) and potentially this might have been the only thermophilic phylotype that was thriving in these 80°C geothermal waters. In contrast, mesophilic KF phylotypes were probably introduced to the sampling site from nearby sources (e.g., banks of outflow channel, groundwater) and thus did not represent the living bacterial community in the KF geothermal water. Note that the mesophilic KF phylotypes were not submitted to GenBank and were excluded from the following discussion.

Each of the remaining four study sites (i.e., GY1, GY2, HV and SV) was characterised by a distinct bacterial community structure each being dominated by one phylogenetic class, which represented between 49 and 95% of the total clone library (Fig. 3). The geochemical parameters that varied most between these four sites were temperature (42–96°C), salinity (<0.1–2.6%) and sinter growth rates (0.2–9.7 kg year^{-1} m^{-2}).

The effect of temperature on bacterial diversity is best exemplified by comparing the bacterial clone libraries for GY1, GY2 and HV (Fig. 3). All these sites were characterised by very similar water chemistries and low sinter growth rates ($\leq$2 kg year^{-1} m^{-2}); the main difference being temperature (Fig. 1, table). The highest bacterial diversity, i.e., highest Shannon–Weaver index (*H*), was found at HV (1.92) and the smallest at GY1 (0.23), where the maximum temperature was about 20°C higher than at HV (Fig. 5). In contrast, at GY2 (76–82°C) a much higher *H* index (1.08) suggested a higher diversity compared to GY1, but still a notably smaller diversity than at HV. These findings indicated an inversely proportional linear trend between the Shannon–Weaver index and temperature (dotted line in Fig. 5). This was further supported by the diversity pattern found at other alkaline, silica-depositing hot springs from Iceland and YNP (USA) (with very similar water chemistries as at GY1, GY2 and HV) which also fell on this linear trend (Fig. 5).

Temperature appeared to also affect the species composition of the bacterial communities at these three sites. Despite some common traits in class-level diversity (Fig. 3), distinct differences were observed at the genus level; GY1, GY2 and KF *Aquificae* clones all branched in the genus *Thermocrinis*, whereas HV clones affiliated to two different lineages, *Sulfurihydrogenibium* and *Thermocrinis* (Fig. 4). This revealed a higher diversity of the *Aquificae* clones at HV (i.e., at lower T), but also suggested that species belonging to *Sulfurihydrogenibium* were confined to temperatures $\leq$74°C (i.e., max. temperature at HV). Similar observations were made by Skirnisdottir et al.

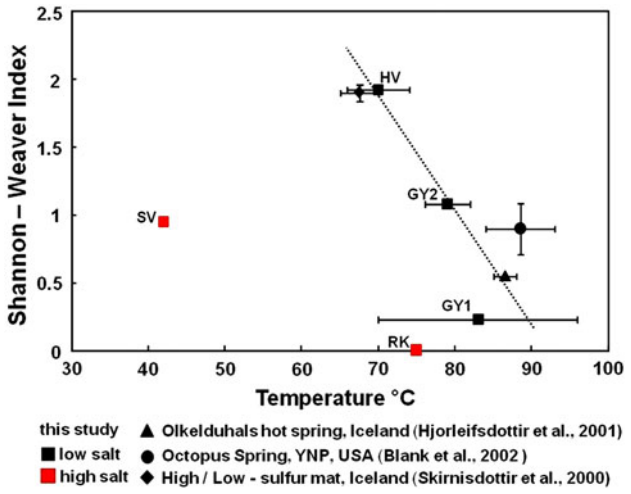

Fig. 5 Water temperature versus Shannon–Weaver index for each site in the current study compared with three hot spring microbial communities from the literature. For hot springs with alkaline, low salt waters (*black symbols*), an inverse linear trend (*dotted line*) between T and the Shannon–Weaver index is evident

(2000), who also studied the bacterial diversity in an adjacent HV spring. However, they concluded that the composition of the *Aquificae* clones at the HV site they studied was influenced not only by temperature but also by varying sulphide concentration. They showed that members of the genus *Thermocrinis* were more dominant in high-T (84–88°C) and low sulphide springs (0.2–1.7 ppm) whilst *Sulfurihydrogenibium* affiliated clones were more abundant in low-T (52–72°C) and high sulphide (3–12 ppm) springs. Although sulphide concentrations were not evaluated in the present study, the presence of elemental sulphur in the in situ grown sinters at HV (Tobler et al. 2008) suggested higher dissolved sulphide values at HV (low-T) than at GY1, GY2 and KF and the results presented here thus agreed with the interpretations of Skirnisdottir et al. (2000).

Similar to the *Aquificae*, the occurrence and diversity of *Deinococci* clones might have been affected by temperature; *Deinococci* clones were the dominant bacteria found at GY2 and HV, but were absent at GY1 and KF. All detected *Deinococci* clones closely related to *Thermus* species which seem to be ubiquitous in most Icelandic hot springs with slightly acidic to alkaline pH (up to pH 10) and temperatures between 60 and 99°C (Hreggvidsson et al. 2006). Although the measured temperature at GY1 was within the T-range favourable for the growth of *Thermus* species, the frequent (<1 min) temperature fluctuations from 70 to 96°C might have been adverse to the colonisation of *Thermus* species. Conversely, at KF, both temperature and pH were stable and favourable for the growth of *Thermus* species, albeit at their upper limit. Therefore, the absence of *Thermus* species at this site might have been due to other geochemical variables not measured here.

At SV, the number of detected OTUs and the Shannon–Weaver index were significantly lower than one would have expected from the linear trend in Fig. 5. This suggested that at this site most likely the limiting factor in terms of bacterial diversity was the high concentration of colloidal silica nanoparticles in the geothermal waters (thickness of sinter accumulation ~0.2 mm per day, Tobler et al. 2008). It is worth pointing out that the SV sampling site was subjected to temperature variations (i.e., temperature increased from 42 to 60°C between September 2005 and July 2007; Tobler et al. 2008) which might have also influenced the developing bacterial communities at this site.

These observations suggested that the presence or absence of certain phylotypes was directly controlled by the geochemical and hydrodynamic regime of the studied geothermal environment, which included parameters, such as T and sinter growth rate. It is important to note that other parameters including the availability and composition of energy sources (e.g., total sulphide concentration, dissolved oxygen, aqueous H_2) or the availability of various organic substrates (e.g., lactic or pyruvic acid) and possibly geographical and historical factors might have also affected the biodiversity found at these study sites. However, their impact on the bacterial community structure was not assessed here as the available data set did not allow for a thorough enough statistical analyses that could include these parameters.

Comparison to the literature

Multiple investigations have analysed the diversity of microbial mats from the Hengill area (i.e., includes Hveragerdi and other surrounding geothermal fields like Grensdalur). For example, Skirnisdottir et al. (2000) analysed the bacterial diversity of a sulphur mat hot spring ($T = 67$°C, pH 6.7) from the riverbank in Grensdalur and found almost exclusively *Aquificae* (*Aquificae* sequences designated SRI in Fig. 4) and low percentages of clones that affiliated with *Thermodesulfobacteria*, *Deinococci*, *Nitrospira* and *Thermotogales*. These observations agreed well with the results at HV, GY1 and GY2 although *Deinococci* clones were numerically better represented than *Aquificae* (except at GY1 where *Deinococci* were absent, Fig. 4). In addition, clones related to *Nitrospira* were also found at HV, however, members of the *Thermodesulfobacteria* and *Thermotogales* were absent at all three sites. It should be noted that compared to the sulphur mat hot spring ($T = 67$°C, pH 6.7), both temperature and pH were higher at the two Geysir sites ($T = 79$–83°C, pH ~ 9), whereas HV featured very similar temperatures, but also a

higher pH ($T = 70$°C, pH ~9). Furthermore, Skirnisdottir et al. (2000) analysed the 16S rDNA gene sequence diversity in microbial mats whilst in the current study (i.e., GY1, GY2 and HV), DNA was extracted from precipitates collected from the bottom of outflow channels/pools (adjacent to the slides trays; Tobler et al. 2008). The observed variations in community structures are thus best explained by the difference in T-pH regime and the nature of the samples, although even small geographical variations should be considered (Hjorleifsdottir et al. 2001).

The microbial community structure of filamentous mats in the Hengill area was also characterised by Hjorleifsdottir et al. (2001), who selected a hot spring in Olkelduhals which had a temperature of 85–88°C, pH 6.9 and abundant filamentous mats. They found that all detected bacterial phylotypes belonged to *Aquificae* and *Deinococci*. The most dominant phylotype was closest related to the Icelandic *Aquificales* clone sequences SRI-48 (Skirnisdottir et al. 2000) and pIce1 (Takacs et al. 2001) and also clustered with EM17, the most dominant clone sequence detected in filamentous mats from Octopus spring, YNP, USA. Note that EM17 was later isolated from this spring and described as *Thermocrinis ruber* (Huber et al. 1998, Fig. 4). These findings fit well with observations made at GY1 and GY2 (Fig. 4) where the temperatures were almost equivalent to those at the Olkelduhals springs (Iceland) and Octopus hot spring (YNP, USA). Conversely, the two *Thermus* phylotypes described by Hjorleifsdottir et al. (2001) at Olkelduhals springs were identical to *Thermus scotoductus* and to *Thermus* str. SRI-248 (which closely relates to *Thermus islandicus;* Bjornsdottir et al. 2009), respectively. Similarly, about 73% of the *Thermus* clones identified at HV (in this study) affiliated with *Thermus scotoductus* but significantly less to *Thermus islandicus* (Fig. 4).

A few studies have described the microbial communities in the near-neutral (pH ~7) and saline geothermal waters at Svartsengi and Reykjanes (e.g., Hreggvidsson et al. 2006; Petursdottir et al. 2000; Petursdottir et al. 2009 and references therein). Petursdottir et al. (2009) used cultivation and culture-independent techniques to analyse the temporal variations in microbial community structure of the Blue Lagoon (effluents of the Svartsengi geothermal power plant, $T = 37$°C and the pH = 7.5) in Iceland between 2003 and 2006. Their results indicated that the microbial communities of the Blue Lagoon (BL) are composed primarily of marine species with most clones being closely related to photoautotrophic *Cyanobacteria* and heterotrophic *Alphaproteobacteria*. They further showed that the microbial diversity was relatively low which they explained by the extreme physico-chemical conditions (i.e., dilute geothermal sea water with 2.5% salinity and high silica content). Their findings agreed well with our observations made in the SV samples ($T = 42$°C, pH = 7.7) where the bacterial clone library was dominated by clones closely related to isolates from both marine and saline terrestrial environments. However, the dominant phyla detected in our SV samples were *Gammaproteobacteria* (*Marinobacter sp.*), and compared to the Blue Lagoon (Petursdottir et al. 2000, 2009), far fewer *Cyanobacteria* and *Alphaproteobacteria* were detected in SV (Table 1). Furthermore, the number of detected species within the SV waters (9 OTUs) was significantly lower than at BL (up to 20 OTUs per 16S rRNA clone library). The observed variations in community structure and diversity are best explained by the difference in T regime (BL: 37°C vs. SV: 45°C) and the nature of the analysed samples (BL: water samples vs. SV: water-saturated precipitates).

Petursdottir et al. (2000) and Hreggvidsson et al. (2006) are the only studies that reported on isolated microorganisms from the very saline, silica-precipitating geothermal waters at Reykjanes (RK). When compared with the RK site sampled in this study, these studies were able to isolate specific species (e.g., *Thermus* sp. and *Rhodothermus marinus* sp.) from waters characterised by a slightly higher salinity (up to 5.8 compared with 4.7% in our RK site), which were also generally cooler (55–70°C, compared with 75°C) and of a lower pH (6.6–6.8 compared with 7.5). Unfortunately, no other geochemical, hydrodynamic or sinter growth rate information for the sites discussed in Petursdottir et al. (2000) and Hreggvidsson et al. (2006) is available. Thus, the question remains open whether a sinter growth rate as high as 300 kg m^{-2} $year^{-1}$ can limit or totally prevent microbial activity as suggested by the RK results presented here, or whether the indigenous microbial communities could simply not be detected by the 16S rRNA gene analysis applied here. It should, however, be noted that both Petursdottir et al. (2000) and Hreggvidsson et al. (2006) stated that the microbial diversity was poor in their samples from Reykjanes. This again highlights the importance of using both cultivation and culture-independent methods but equally shows that it is essential to measure the surrounding geochemical and hydrodynamic conditions to be able to make conclusions on microbial ecology.

Conclusions

The data presented here suggested that the bacterial diversity in silica precipitates from six different Icelandic geothermal sites varied with temperature, but other factors like sinter growth rate also influenced the bacterial community structure. As such, it was not possible to single out one parameter that affected the microbial community

structure in all sites but it is clear that the biodiversity patterns determined at each site was controlled by a combination of these parameters. In this study, the most extreme habitat was defined by the combination of high temperatures (≥75°C), high salinity (≥4.7%) and high sinter growth rates (≥300 kg year^{-1} m^{-2}), but at this site neither bacteria nor archaea were found (i.e., RK). These results further indicated that the physico-chemical conditions defining the precipitation of amorphous silica (i.e., sinter growth rates, Tobler et al. 2008) exert a strong control on the microbial ecology and distribution.

The comparison to other molecular studies on bacterial diversity in alkaline, silica-precipitating hot springs showed that the dominant phylotypes fall mainly into the same phylogenetic classes (i.e., *Aquificae*, *Deinococci*, *γ-Proteobacteria*). Furthermore, some phylotypes (e.g., *Thermus* spp., *Thermocrinis* ssp.,) were found in a variety of hot springs indicating that they can adapt to different geochemical/hydrodynamic regimes.

Overall, we confirmed that in geothermal areas the physico-chemical characteristics invariably affect the diversity and structure of microbial communities. However, this study also revealed that only by exploring these links in as many diverse sites as possible via the full geochemical, physical and microbial analyses can a deeper insight into the complexity of geothermal microbial communities and their broader relevance at a global scale be derived.

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